RESEARCH COMMUNICATION

Physiological control of metabolic flux: the requirement for multisite modulation

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Biochemists have long assumed that the flux through a metabolic pathway can be controlled by the activity of a key regulatory enzyme near the beginning of the pathway. We present the accumulating evidence that every step in this assumption is flawed. Instead, effective physiological control of metabolism is shown to involve simultaneous multisite modulation through action on a number of enzymes.

INTRODUCTION

Since Blackman [1] proposed the concept of the 'rate-limiting step' in 1905, it has dominated the approach to understanding the control of metabolic pathways. For example, it was endorsed in Krebs' concept of 'pacemaker' enzymes [2], which he saw as the target sites for hormone and drug action on metabolism. Even though the theory of Metabolic Control Analysis [3,4] has since shown that control can be distributed over many steps in a pathway, and that the degree of control of any given step can be quantified by its flux control coefficient, qualitative explanations of how a pathway can be controlled have not been greatly affected. Indeed, although Metabolic Control Analysis has been increasingly adopted in metabolic biochemistry, and experiments have confirmed both that control is generally distributed [5] and that genuinely rate-limiting enzymes are rare, it has also legitimized the concept that an enzyme that responds to some external controlling factor can be an agent of metabolic control provided the enzyme has a finite flux control coefficient. However, we shall cite arguments that such mechanisms cannot be responsible for large changes in metabolic flux. On the other hand, recent theoretical developments arising from Metabolic Control Analysis do allow us to characterize how large changes in metabolic flux could be implemented; they can only be achieved with minimal disturbance of metabolite concentrations and fluxes in other pathways by co-ordinated changes in the activities of many of the enzymes in the pathway, and this can be shown to be a common mechanism of control.

Related experimental and theoretical evidence also contradicts the view that regulatory enzymes exhibiting allosteric properties are effective agents for control of metabolic flux. Our conclusion is that their more significant role is in homoeostasis. In consequence, different approaches are needed to both the study and explanation of metabolic control.

LIMITS ON SINGLE ENZYME ACTIVATION

The question of how effectively the flux in a metabolic pathway can be increased through increasing the activity of a single enzyme has been addressed by Small and Kacser's theory [6,7] of finite changes. Their approximate solution for the factor f by which the pathway flux will increase for an r-fold increase in the amount of enzyme activity in a linear pathway is:

$$f = \frac{1}{1-(r-1/r)\,C_E^J} \qquad (1)$$

where C_E^J is the flux control coefficient of the enzyme E on the pathway flux, J. {The flux control coefficient can be simply regarded as the percentage change in flux caused by a percentage change in enzyme activity [3], but in the limit of vanishingly small changes, not the large changes considered in eqn. (1).} This function is plotted in Figure 1, where it is seen that the effects on the pathway flux from changing the amount of a single enzyme can be quite limited, unless its flux control coefficient is greater than 0.6. Experimental Control Analysis has confirmed the theoretical expectation that individual steps in metabolic pathways generally do not have large flux control coefficients [5]. Additional experimental support comes from the successful use of molecular genetic techniques to increase the amount of single target enzyme in cells, including the 'regulatory enzyme' phosphofructokinase: the effects on metabolic flux have often been small or non-existent [8–12]. Therefore control of the flux in a metabolic pathway by action on a single regulatory enzyme is likely to be relatively ineffective and limited to small changes in flux. (Strictly, this conclusion applies to increasing the flux, since flux in a pathway can always be decreased to any degree by a sufficient decrease in the activity of anyone of its steps, though this may cause extensive perturbation of metabolite concentrations.) However, to achieve a relatively large change in flux in the absence of any one enzyme having a flux control coefficient in the region of 0.6 (or above), it is possible to increase a number of enzymes simultaneously (by the factor predicted by eqn. 1) if they are chosen so the sum of their coefficients gives the desired high group coefficient [6]. The analysis can also be generalized to branched pathways [7].

PROBLEMS IN THE CONTROL OF FLUX BY FEEDBACK INHIBITION

Doubts have recently been growing about the importance of allosteric effectors in flux control [13–16]. Certainly, in amino acid biosynthesis in bacteria, there are clear examples where the allosteric effects implementing feedback inhibition have been

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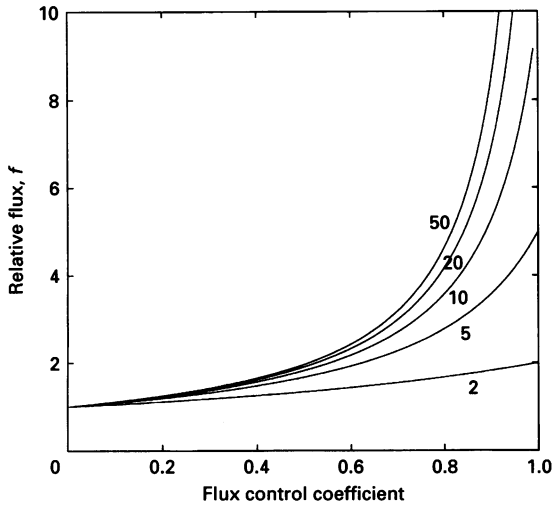

Figure 1 Relative change of flux for large changes in enzyme amount

The increases in flux predicted by eqn. (1) are plotted against the value of the flux control coefficient [6]. The degree of amplification, r of the enzyme amount is shown on each curve.

demonstrated to control biosynthetic flux, but there are also others where the evidence for physiological relevance either is lacking or is undermined by the absence of the expected phenotypic differences for mutants in the regulatory properties of the allosteric enzyme [14,17]. In other pathways, biochemists ascribe metabolic control to allosteric regulation with little quantitative evidence, but there are anomalies. In carbohydrate metabolism, mutants for allosteric enzymes that have altered regulatory properties either show no substantial changes in flux and metabolite concentrations, or show changes that are difficult to interpret [13–15]. The aerobic–anaerobic transition in yeast is accompanied by a clear 'cross-over' at phosphofructokinase [18], indicative of the action of an allosteric effector accompanying the flux change, yet it is known that this enzyme cannot exert much control [8,10].

Theoretical studies of feedback inhibition have revealed many interesting properties of this form of allosteric regulation, but little support for the concept that its primary role is the control of flux. On the contrary, at the outset of Metabolic Control Analysis, Kacser and Burns [3] concluded that the effect of feedback inhibition would be to lower the flux control coefficient of the inhibited enzyme and to transfer control to the steps downstream from the feedback loop. This prediction has been fully vindicated by the lack of effects on flux from over-expression of allosterically regulated enzymes. Savageau [19] showed that feedback inhibition lowers the variation in metabolite concentrations within the loop in response to changes in the input metabolite or in the net rate of utilization of the inhibitory metabolite. Feedback inhibition has been shown theoretically to increase the stability of a pathway by increasing its rate of return to a steady state after a perturbation [20], and to decrease the transition time for a pathway to change to another metabolic state [21].

Hofmeyr and Cornish-Bowden [22] showed that flux control exerted at the beginning of a pathway is incompatible with effective homoeostasis of intermediates. Low sensitivity of intermediates to changes in flux rates is only obtained when the control of flux is exercised by the steps downstream from these metabolites, as is likely to happen when there is a feedback-inhibition loop on to an early enzyme in the pathway. A high degree of co-operativity of feedback effects gives a significant improvement in homoeostasis of the concentration of the feedback metabolite, but is of lesser importance for the effective transfer of flux control to the downstream steps. The relative constancy of glycolytic intermediates in muscle upon large changes in glycolytic flux (see below) therefore excludes the possibility that this flux change is initiated solely by enzymes at the beginning of the pathway.

These findings combine to give a theoretical basis to Bücher and Rüssman's [23] suggestion that homoeostasis of metabolite concentrations is an important aspect of metabolic regulation, ensuring what they called 'functional readiness'.

Another conventional concept about allosteric control is that it is responsible for rapid responses in flux control on shorter time-scales than covalent modification or enzyme synthesis and degradation [24,25]. However, the transition time for pathways to reach a steady-state flux is typically in the region of seconds to minutes [20,21,26], which is the time-scale on which covalent modification works. Thus there is no obvious 'functional gap' for allosteric effects to fill in flux control whenever covalent modification mechanisms are available.

We can distinguish between regulation, the maintenance of a uniform internal milieu in the face of environmental changes, and control, the ability to change the metabolic state [5,22]. On this basis, the evidence is that allosteric enzymes promote regulation (not control) in that they are homoeostatic mechanisms ensuring rapid stabilization of pathways and regulation of metabolite levels, and are of limited significance for flux control, except perhaps for inhibition of some biosynthetic pathways in prokaryotes in the absence of alternative mechanisms.

LIMITS ON THE ADAPTABILITY OF NEAR-EQUILIBRIUM ENZYMES

Implicit in the traditional concept that metabolic flux might be controlled at a small number of regulatory enzymes is the expectation that other enzymes, particularly near-equilibrium enzymes, can passively transmit a change in flux along the chain solely *via* changes in their substrate and product concentrations. For a reaction to be sufficiently near equilibrium to allow this, eqns. (A6) and (A7) presented in the Appendix demonstrate the stringent requirement that is placed on the lower limit of the disequilibrium ratio ρ (that is, the ratio of the mass-action ratio to the equilibrium constant, or $\Gamma/K_{eq.}$) if the relative increase in substrate concentration is less than the relative increase in pathway flux. The disequilibrium ratio for a single near-equilibrium step would need to be greater than 0.99 in a pathway such as muscle glycolysis, where flux can increase over 100-fold with less than 10-fold changes in the concentrations of substrates and products [27–29]. Many enzymes classified as near-equilibrium steps by convention (e.g. $\rho > 0.2$) will not be sufficiently near on these criteria.

REQUIREMENT FOR MULTISITE MODULATION

If pathway flux cannot be activated many-fold by control of a single, or even a few, enzymes near the beginning of a pathway, what is the alternative? Metabolic control through action on several enzymes was first suggested by Bücher and Rüssman [23] in 1963. They had noted that large changes in animal muscle glycolysis were accompanied by relatively insignificant changes in the proportions of the metabolic intermediates [27], and concluded that there must therefore be several controlling

reactions. Helmreich and Cori [28] also thought that muscle glycolysis must be controlled as a unit in order to explain similar results from their experiments on stimulation of muscle. Metabolic Control Analysis and related theory now offer the means to re-examine these proposals. Kacser and Acarenza [30], in their 'Universal Method', proposed how biotechnologists might design a change in metabolism to cause an arbitrarily large flux increase in a specific part of metabolism, whilst leaving all concentrations and most other fluxes unperturbed. For simplicity, assume the target pathway is one producing an end-product of metabolism. The method proposes making proportional increases in all the enzymes leading directly to this output from the input(s), with the largest changes in activity being made in all the enzymes in the final linear sequence to the output, and with progressively smaller changes being made in each preceding branch so that the fluxes in lateral branches remain unchanged. In effect, the method exploits the different behaviour of fluxes and concentrations when the activities of a sequence of enzymes are all increased in the same proportion. According to the summation theorem for flux control coefficients [3], the flux will increase in proportion to the enzyme activities, whereas the metabolite concentrations will remain constant in accordance with the summation theorem for concentration control coefficients [4]. It might not be necessary to increase every enzyme in any particular branch provided that any enzymes omitted have near-zero flux control coefficients, and that the sum of the flux control coefficients of the changed enzymes is close to 1. (Any unchanged enzymes would have to be near-equilibrium by the stringent conditions of the Appendix.) The potential for success of this strategy of multiple enzyme manipulations, compared with the failure of manipulating single enzymes in a pathway, has been illustrated with the tryptophan biosynthesis pathway of yeast [11]. An important aspect of the results was the demonstration that the flux increase achieved when all the enzymes were changed together was far greater than the product of the flux changes obtained by increasing each enzyme separately.

CHARACTERISTICS OF CONTROL BY MULTISITE MODULATION

If the principles of the Universal Method do show how physiological control by multisite modulation could work, we should observe the following characteristics in its operation *in vivo*.

(1) When enzyme amounts change in response to physiological or environmental signals, the relative proportions of pathway enzymes remain constant.
(2) The common factor by which the amounts of the pathway enzymes change is equal to the factor by which the flux changes.
(3) The levels of change are greatest in the main branch of the pathway being controlled, although co-ordinated, but smaller, changes occur in more distal branches.
(4) Apart from enzyme induction, other control mechanisms that act on the pathway also operate on a similar set of multiple target sites.

The first point is well illustrated by the organization of pathway enzymes in operons, as adjacent genes with a common control of expression, and also by 'regulons' [31], where the genes are not all adjacent and do not share control elements, yet still respond to the same signals. Srere [32] has recently noted that the concept of control by rate-limiting steps is contradicted by the many examples (glycolysis, the tricarboxylic acid cycle, photosynthesis and the syntheses of fatty acids, urea, nucleotides and amino acids) where environmental or physiological signals cause co-ordinated induction of all the enzymes in a pathway. We shall therefore concentrate on examples that illustrate the other three characteristics.

Lipogenesis exemplifies all three. The mouse *obese* gene codes a secreted protein, specific to adipose tissue, that appears to have signalling functions and is responsible for profound obesity [33]. The enzymes whose activities are known to change in the *obese* phenotype [34] substantially overlap with those enzymes along the pathways from carbohydrate to lipid, for which there is good evidence of transcriptional control of expression responsive to starvation, refeeding or high-carbohydrate diets [35] in rats. The mutant mouse must be capable of a rate of lipid synthesis per unit of lean tissue some 3-fold higher than in normal mice [34], and this is consistent with eight of the major enzymes of lipid and carbohydrate metabolism showing activities 2–4-fold higher than normal (point 2). In connected parts of carbohydrate metabolism, four enzymes are elevated about 50%, and two adipose-tissue lipases are significantly depressed [34] (point 3). Four of the enzymes along this route are known to be subject to control by protein phosphorylation and dephosphorylation reactions that respond to dietary status [35] (point 4). However, the evidence for involvement of allosteric effectors in physiologically significant dietary control of lipogenesis is weaker [35].

The rate of urea synthesis in rats responds proportionately to the amount of protein in the diet, and, over a range of protein intakes, the amounts of the urea-cycle enzymes also vary proportionately [36]. A comparison of the enzyme contents of rats on diets causing a 4-fold difference in urea outputs showed that eight of the measured enzymes increased significantly. All the enzymes of the urea cycle increased 2–3-fold in activity, which is sufficient to account for the flux change if there is also a slight stimulation by increased substrate supply (point 2 above). There were also large increases in alanine transaminase and glucose-6-phosphate dehydrogenase.

In the case of glycolysis and gluconeogenesis in mammalian liver, Weber and his colleagues [37,38] concluded from their studies on the effects of diet, starvation and hormone treatments that the enzymes fall into three groups: the exclusively gluconeogenic enzymes, the exclusively glycolytic enzymes, and the bifunctional enzymes. The amounts of members of a group change co-ordinately, but the directions and magnitudes of the changes differ between the groups, exemplifying points 1 and 3 above. For several of these enzymes, the molecular mechanisms of control of enzyme amount have been shown to include the actions of insulin, cyclic AMP and glucocorticoids on transcription [39,40]. Some support for a similar pattern of control by other mechanisms (point 4) comes from the effects of protein phosphorylation. The actions of glucagon and α-adrenergic agents in stimulating gluconeogenesis in rat liver involve inhibition of pyruvate kinase by phosphorylation, and the activation of fructose-1,6-bisphosphatase and the inhibition of phosphofructo-1-kinase by changes in the level of fructose 2,6-bisphosphate brought about by protein phosphorylation. As it is possible that as many as one in four mammalian cell proteins can be reversibly phosphorylated [41], it is conceivable that there are more undiscovered sites of phosphorylation involved in the stimulation of gluconeogenesis. Interpretation is complex because, in other tissues, protein phosphorylation stimulates glycolysis. For example, in different types of muscle, stimulation of contraction may be associated with activation of some or all of phosphorylase, phosphofructo-1-kinase, pyruvate dehydrogenase and myosin ATPase.

The acute and chronic stimulations of the synthesis of corticosteroid hormones in the adrenal cortex by corticotropin provide another example of parallel multisite changes in both control of enzyme amount and more rapid modulation of activity by

mechanisms including protein phosphorylation. Both involve action on all aspects of metabolism affecting the intracellular cholesterol pool, its conversion into the common intermediate pregnenolone, and on many sites in the final formation of products by oxidation [42,43]. In other related branches of metabolism, esterification for storage in lipid droplets is inhibited while increased activities of other enzymes increase the rate of supply of precursors for cholesterol synthesis [44] and reducing equivalents for the hydroxylation reactions [43].

Finally, light-dependent activation of plant photosynthesis and associated metabolism illustrates rapid control mechanisms acting at multiple sites throughout a pathway (point 4). As well as the diurnal cycle, plants are subject to continual fluctuations in illumination from light flecks etc. There can be responsible for a significant fraction of daily carbon fixation and can cause increases in assimilation of CO_2 of the order of 10-fold on a time scale of a few seconds. The activation state of four enzymes of the Calvin cycle depends on light-mediated reduction via thioredoxin that is also modulated by metabolite levels [45]. Calvin-cycle enzymes also respond to light-induced changes in stromal pH and Mg^{2+} concentration [46]. Ribulose-bisphosphate carboxylase (rubisco) is controlled by multiple effects, including light-dependent carbamoylation by rubisco activase [45] and, in C_4 plants, the activity of pyruvate,phosphate dikinase is controlled by phosphorylation in a light-dependent manner [47]. In addition, light-dependent changes in protein phosphorylation activate enzymes of the assimilatory pathways in the cytoplasm, including sucrose phosphate synthase, nitrate reductase and phosphoenolpyruvate carboxykinase [47].

CONCLUSIONS

The importance of the concept of control by multisite modulation is that it leads to a radical reinterpretation (at complete variance with textbook concepts of metabolic control) of results that have been accumulating in experimental biochemistry for the past 30 years. In future, a different approach must be taken to the experimental study of control mechanisms in metabolism and signal transduction: biochemists should widen their search for controlling enzymes, rather than focussing on the minimum possible number of candidates. Our findings also explain failures to influence metabolism by genetic manipulation. Finally, this new view highlights a continuing problem in metabolism: if control is achieved by synergistic interaction between multiple control sites, none of which separately can account for the observed effects, then control of metabolic systems can only be understood at the system level, and the effects of varying a single component in isolation will underestimate its contribution when several components are varied simultaneously. Interpretation of experimental results will require the use of theoretical tools such as mathematical modelling and the latest developments of Metabolic Control Analysis [6,7,30], including Modular Control Analysis [48], to validate proposed control mechanisms quantitatively.

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REFERENCES

1 Blackman, F. F. (1905) Ann. Bot. (London) **19**, 281–295
2 Krebs, H. A. (1957) Endeavour **16**, 125–132
3 Kacser, H. and Burns, J. A. (1973) Symp. Soc. Exp. Biol. **27**, 65–104
4 Heinrich, R. and Rapoport, T. A. (1974) Eur. J. Biochem. **42**, 89–95
5 Fell, D. A. (1992) Biochem. J. **286**, 313–330
6 Small, J. R. and Kacser, H. (1993) Eur. J. Biochem. **213**, 613–624
7 Small, J. R. and Kacser, H. (1993) Eur. J. Biochem. **213**, 625–640
8 Heinisch, J. (1986) Mol. Gen. Genet. **202**, 75–82
9 Schaaff, I., Heinisch, J. and Zimmerman, F. K. (1989) Yeast **5**, 285–290
10 Davies, S. E. C. and Brindle, K. M. (1992) Biochemistry **31**, 4729–4735
11 Niederberger, P., Prasad, R., Miozzari, G. and Kacser, H. (1992) Biochem. J. **287**, 473–479
12 Burrell, M. M., Mooney, P. J., Blundy, M. et al. (1994) Planta **194**, 95–101
13 Fraenkel, D. G. (1986) Annu. Rev. Biochem. **55**, 317–337
14 Fraenkel, D. G. (1992) Annu. Rev. Genet. **26**, 159–177
15 Manjrekar, J. (1993) Curr. Sci. **65**, 443–447
16 Srere, P. A. (1994) Trends Biochem. Sci. **19**, 519–520
17 Umbarger, H. E. (1978) Annu. Rev. Biochem. **47**, 533–606
18 Lagunas, R. and Gancedo, C. (1983) Eur. J. Biochem. **137**, 479–483
19 Savageau, M. A. (1974) J. Mol. Evol. **4**, 139–156
20 Reich, J. G. and Sel'kov, E. E. (1981) Energy Metabolism of the Cell, Academic Press, London
21 Easterby, J. S. (1986) Biochem. J. **233**, 871–875
22 Hofmeyr, J.-H. S. and Cornish-Bowden, A. (1991) Eur. J. Biochem. **200**, 223–236
23 Bücher, T. and Rüssman, W. (1964) Angew. Chem., Int. Ed. Engl. **3**, 426–439 (originally published as Angew. Chem. **73**, 881 in 1963)
24 Ashworth, J. M. and Kornberg, H. L. (1963) Biochim. Biophys. Acta **73**, 519–522
25 Krebs, E. G. (1986) Enzymes 3rd Ed. **17**, 1–20
26 Torres, N. V. and Meléndez-Hevia, E. (1992) Mol. Cell. Biochem. **12**, 109–115
27 Hohorst, H. J., Reim, M. and Bartels, H. (1962) Biochem. Biophys. Res. Commun. **7**, 137–141
28 Helmreich, E. and Cori, C. F. (1965) Adv. Enzyme Regul. **3**, 91–107
29 Sacktor, B. and Wormser-Shavit, E. (1966) J. Biol. Chem. **241**, 624–631
30 Kacser, H. and Acarenza, L. (1993) Eur. J. Biochem. **216**, 361–367
31 Maas, W. K. and Clark, A. J. (1964) J. Mol. Biol. **8**, 365–370
32 Srere, P. A. (1993) Biol. Chem. Hoppe-Seyler **374**, 833–842
33 Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J. (1994) Nature (London) **372**, 425–432
34 Bulfield, G. (1972) Genet. Res. (Cambridge) **20**, 51–64
35 Hillgartner, F. B., Salati, L. M. and Goodridge, A. G. (1995) Physiol. Rev. **75**, 47–76
36 Schimke, R. T. (1962) J. Biol. Chem. **237**, 459–468
37 Weber, G., Singhal, R. L. and Srivastava, S. K. (1965) Adv. Enzyme Regul. **3**, 43–75
38 Weber, G., Singhal, R. L., Stamm, N. B., Lea, M. A. and Fisher, E. A. (1966) Adv. Enzyme Regul. **4**, 59–81
39 Pilkis, S. J. and Claus, T. H. (1991) Annu. Rev. Nutr. **11**, 465–515
40 Lemaigre, F. P. and Rousseau, G. G. (1994) Biochem. J. **303**, 1–14
41 Chelsky, D., Ruskin, B. and Koshland, D. E., Jr. (1985) Biochemistry **24**, 6651–6658
42 Pedersen, R. C. and Brownie, A. C. (1986) Biochem. Actions Horm. **13**, 129–166
43 Waterman, M. R. and Simpson, E. R. (1989) Recent Prog. Horm. Res. **45**, 533–566
44 Kowal, J. (1970) Recent Prog. Horm. Res. **26**, 623–687
45 Geiger, D. R. and Servaites, J. C. (1994) Annu. Rev. Plant Physiol. Plant Mol. Biol. **45**, 235–256
46 Woodrow, I. E., Murphy, D. J. and Latzko, E. (1984) J. Biol. Chem. **259**, 3791–3795
47 Huber, S. C., Huber, J. L. and McMichael, R. W. (1994) Int. Rev. Cytol. **149**, 47–98
48 Kahn, D. and Westerhoff, H. (1991) J. Theor. Biol. **153**, 255–285

APPENDIX

Consider a single-substrate, single-product enzyme obeying the reversible Michaelis–Menten rate equation:

$$v = \frac{(V_{m,f}/K_{m,S})(S - P/K_{eq.})}{1 + S/K_{m,S} + P/K_{m,P}} \quad \text{(A1)}$$

where v is the net rate (positive for formation of P), $V_{m,f}$ is the maximum velocity in the forward direction, $K_{m,S}$ and $K_{m,P}$ are K_m values for substrate and product, $K_{eq.}$ is the equilibrium constant for the reaction, and S and P are the metabolite concentrations. On activation of the pathway containing this

enzyme, the net flux is increased M-fold, so that the new rate v_a is given by:

$$v_a = Mv \tag{A2}$$

and the new metabolite concentrations are increased r- and t-fold:

$$S_a = rS;\ P_a = tP \tag{A3}$$

If we consider the case where the denominator of eqn. (A1) remains unchanged near 1 (i.e., the enzyme is unsaturated, but see below) then:

$$\frac{V_a}{v} = M = \frac{rS - tP/K_{eq.}}{S - P/K_{eq.}} \tag{A4}$$

Rearranging for t and introducing the definition of the disequilibrium ratio, $\rho = P/(SK_{eq.})$ gives:

$$t = M - \frac{M-r}{\rho} \tag{A5}$$

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If there has been no decrease in P, i.e. $t \geqslant 1$, then eqn. (A5) implies:

$$\rho \geqslant \frac{M-r}{M-1} \tag{A6}$$

If $M \geqslant 100$ and $r \leqslant 10$ (as in stimulation of muscle glycolysis), the minimum value of ρ is 0.91.

If both S and P increase, the new disequilibrium ratio is $\rho_a = pt/r$. Substituting this in eqn. (A5) gives:

$$1 - \rho a = \frac{M}{r}(1-\rho) \tag{A7}$$

If in addition to the previous requirement that $M \geqslant 100$ and $r \leqslant 10$ we require $t \geqslant 0.9r$, to ensure onward transmission of the metabolite increase down the chain, then either eqn. (A5) or (A7) shows $\rho \geqslant 0.99$.

If the enzyme undergoes a significant change in saturation with the flux increase, the lower bounds on the ρ given above are underestimates.